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14. ABSTRACT

The humoral response of a cancer patient may allow earlier detection of cancer than current methods allow. If so, the serum autoantibody repertoire from cancer patients might be exploited for autoantibody profiling, and aid in the serological diagnosis of cancer. In this summary, we report the development of a whole proteome native antigen microarray for human breast cancer. This was accomplished by using a 2-D liquid chromatography fractionation strategy, where the 1st dimension is separation by isoelectric points and the 2nd dimension is separation by hydrophobicity. Using this platform and sera from stage 1 and 2 invasive ductal carcinoma of the breast as well as normal controls, we identified antigen containing fractions that were significantly differentially reactive with the cancer sera ($p \leq 0.05$). Receiver operator characteristics curves were plotted for the top 5 reactive fractions and the area under the curve (AUC) was calculated. Our findings showed that when combined, the 5 reactive fractions have an AUC of 0.898 for stage 1 breast cancers versus normal controls, and an AUC of 0.82 for stage 2 breast cancers versus normal controls. Our preliminary results demonstrate that we have successfully developed a platform for autoantibody profiling, and that there are specific immune response signatures that might serve as potential biomarkers.

15. SUBJECT TERMS

Whole proteome native antigen microarray; biomarkers, autoantibody profiling, breast cancer.

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INTRODUCTION:

Cancer sera contain antibodies that react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs). Proteins not present in normal cells may elicit a host immune response, which affords a dramatic amplification of signal in the form of antibodies relative to the amount of the corresponding antigen. In addition, the humoral response may allow earlier detection of cancer than current methods allow. If so, the serum autoantibody repertoire from cancer patients might therefore be exploited for autoantibody profiling, and potentially aid in the serological diagnosis of cancer. To date, however, studies of antigen-autoantibody reactivity using protein microarrays have relied on recombinant proteins or synthetic peptides as arrayed features. This current approach may fail to accurately detect autoantibody binding due to the lack of proper post-translational modifications and antigen folding. In this report, we report the development of a whole proteome native antigen microarray platform for autoantibody profiling of human breast cancer, and demonstrate its utility in identifying relevant autoantibody signatures for early stage breast cancer.

BODY:

Even with the use of expression systems such as the baculovirus expression system, which renders protein modifications observed with mammalian cells, such post-translational modifications (PTMs) remain dependent of the expression systems that are used and may not reflect the unique modifications that are associated with a human disease. It is also important to note that several studies have shown that PTMs on proteins serve as stimuli or epitopes for autoantibody reactivity (1-3). Thus, the objectives of our research are to: 1) develop a whole proteome native antigen fractionation microarray platform for antigen-autoantibody profiling, and 2) test the hypothesis that patients with breast cancer may elicit a host immune response and that global autoantibody profiling might identify relevant disease signatures as potential biomarkers.

In brief, we developed a whole proteome native antigen fractionation microarray platform for antigen-autoantibody profiling by separating well-characterized tumor cell lysates into defined antigen fractions based on the antigen's individual chemistry. This was accomplished by using a 2-D liquid chromatography fractionation strategy, where the 1st dimension is separation by isoelectric points and the 2nd dimension is separation by hydrophobicity. This strategy gave us over 1,000 fractions with each fraction containing between 1 and 10 different proteins. Following the 2nd dimension, the fractions containing the tumor antigens were arrayed onto nitrocellulose coated microscope slides (see figure 1, below). These spotted fractions contained proteins in their native states and, as such, contained relevant post-translational modifications and alterations that are specific to the disease, i.e., cancer, thereby allowing these arrays to detect autoantibody reactivity to disease-related epitopes. IgGs were purified from well-characterized serum samples from patients with newly diagnosed breast cancer obtained prior to surgery and any treatments and age- and estrous cycle-matched healthy controls. The slides were then probed with the purified dye labeled samples and scanned for fluorescent intensity.

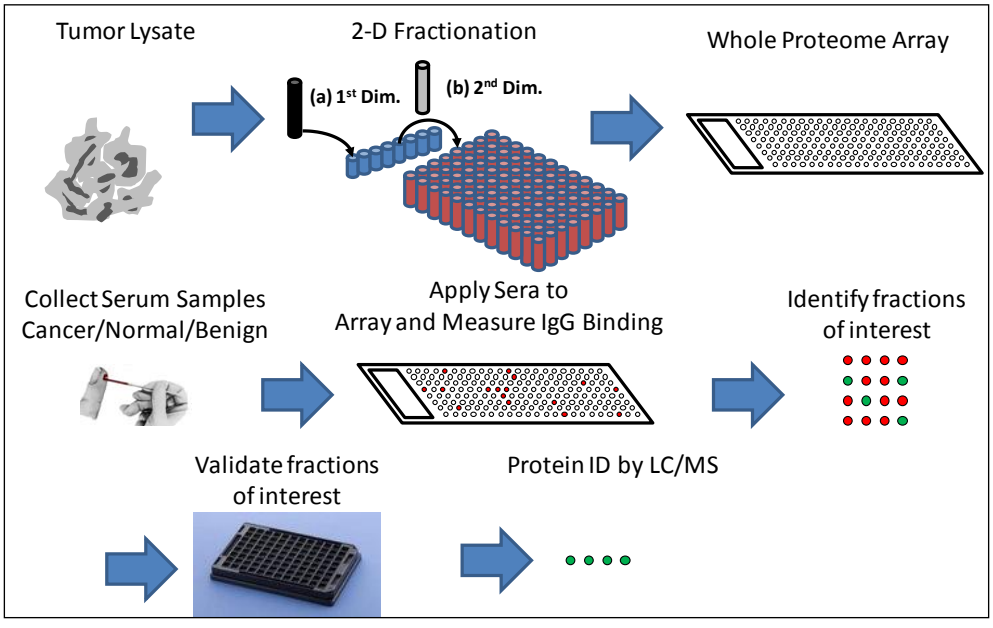

Figure 1: This figure is a schematic showing that the whole proteome from a tumor is fractionated by isoelectric points and hydrophobicity using 2-D liquid chromatography. The different fractions are then printed onto arrays and incubated with labeled serum antibodies. After detection, the informative fractions and associated antigens are identified using mass

spectrometry, and can be further validated by using various techniques such as ELISA and/or tissue microarrays.

For the results presented here, we used a single dye (Cy3) for comparisons between breast cancer and normal controls. IgGs from individual breast cancer patients (n=40; all with invasive ductal carcinoma of the breast) and from age- and estrous cycle-matched controls were purified using the Melon Gel IgG Purification kit (Pierce Biotechnology) and labeled with Cy3 dye. The whole proteome native antigen fractionation microarrays were then probed with individual samples from breast cancer cases and normal controls. Figure 2 (below) illustrates the reactivity in the cancer cases and normal controls.

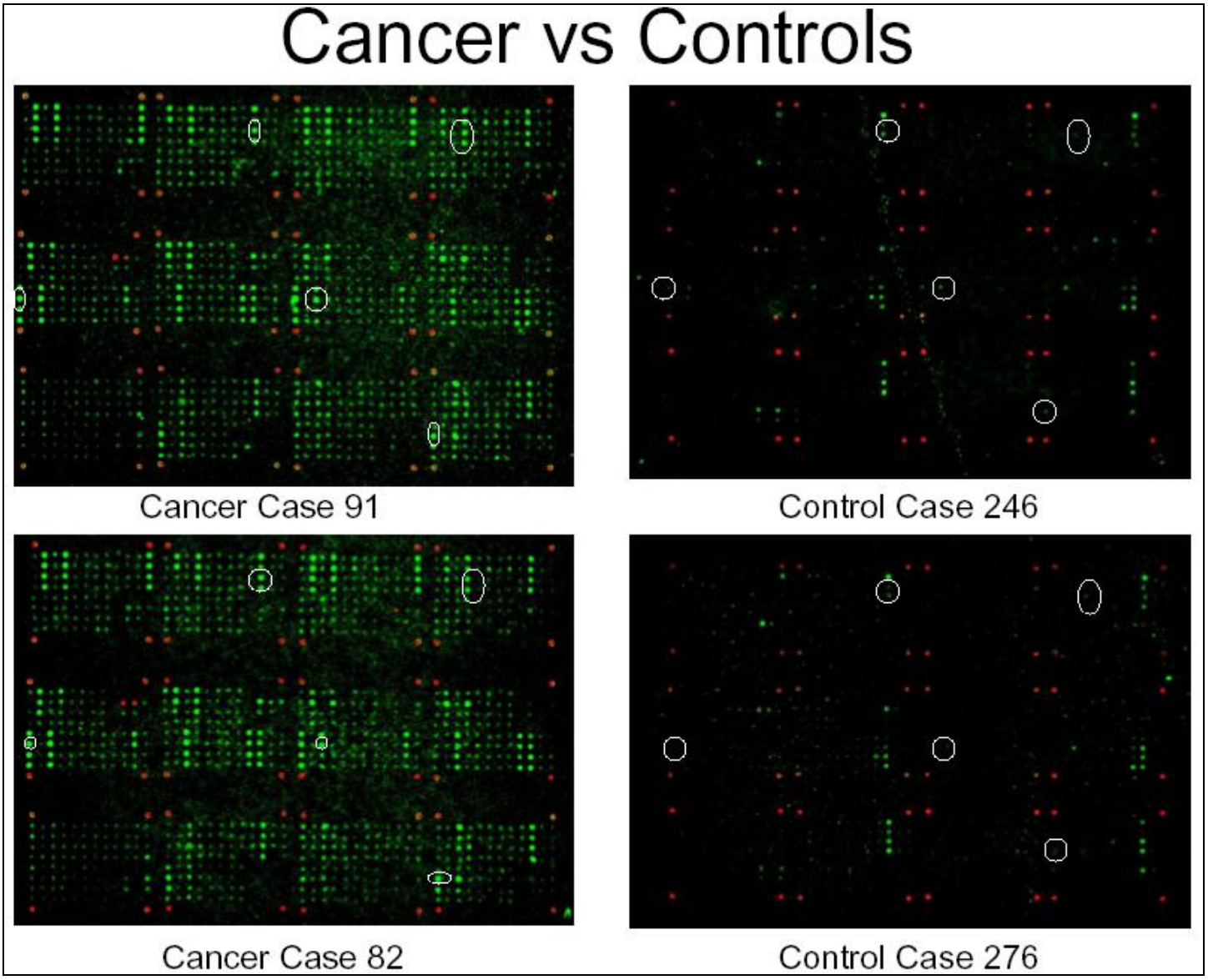

Figure 2: This figure illustrates representative antigen-autoantibody reactivity in 2 breast cancer cases and 2 normal controls. Each array was probed with Cy3 dye-labeled purified IgGs from either the cases or controls. These images demonstrate that there is a high-degree of similarity in autoantibody reactivity to spotted fractions on the whole proteome native antigen microarray between the cancer cases. The same autoantibody reactivity for the arrayed fractions was not detected in the controls. Several fractions are circled to illustrate the strong reactivity in the cancer cases and not in the normal controls.

To correct for intra- and inter-slide variations, a signal-to-noise ratio was computed from the difference of the intensity of each spot minus the background intensity of the spot divided by the standard deviation of the background intensity. Following normalization, Student T-tests were performed and the fluorescence intensities of the significant antigens were used to perform 2-D hierarchical clustering and to construct a heat map that represents sample proximity. Figure 3 below is a heat map of 23 fractions with a statistical significance above 95% across all samples.

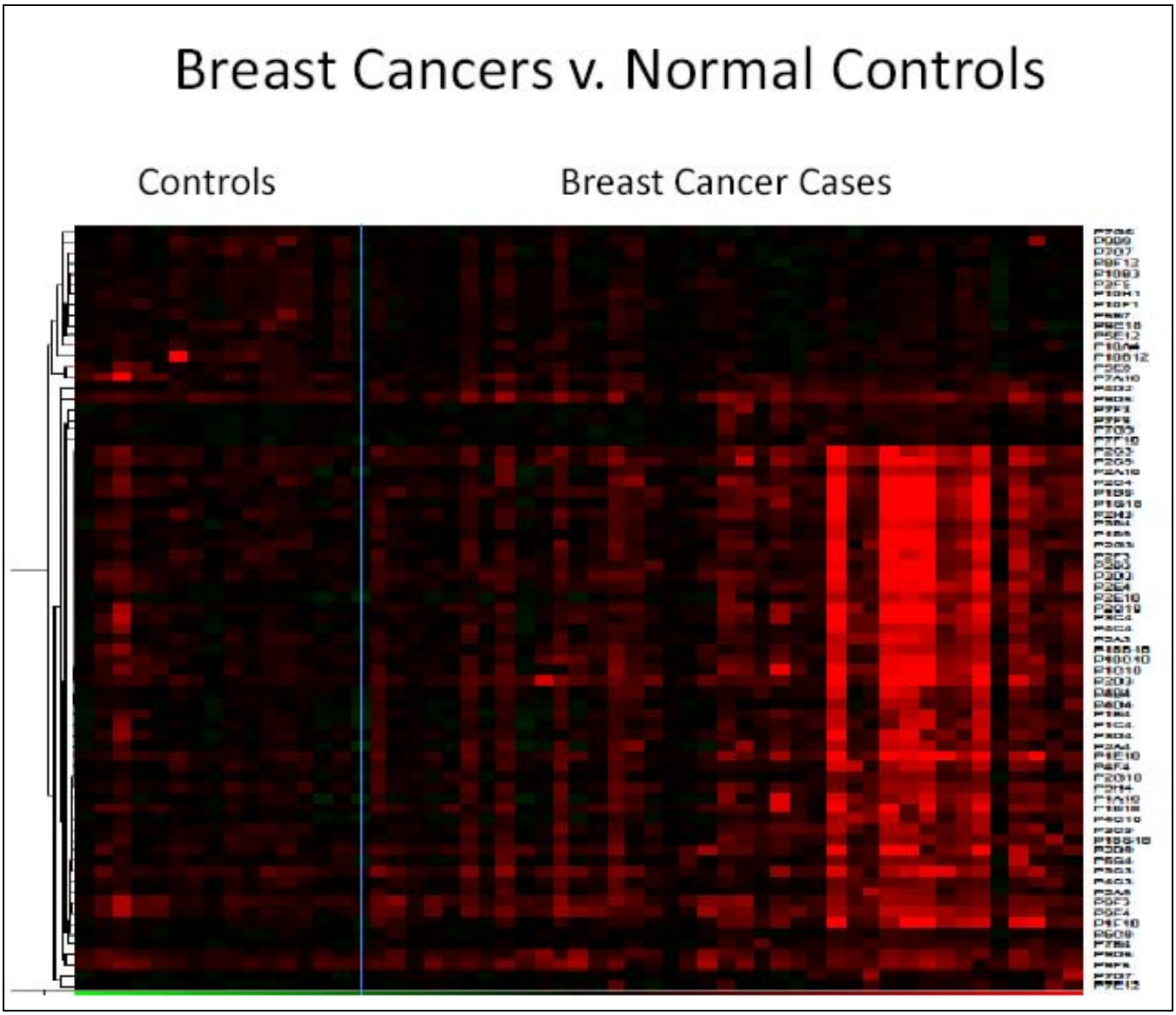

Figure 3: This heat map illustrates the spatial relationship between significant antigen fractions of stage 1 and stage 2 breast cancer cases as well as normal controls. Significant fractions were selected following a Student T-test ($p \leq 0.05$) and clustering was performed using a Euclidean similarity metric. The blue line separates the normal controls on the left from the cancer patients on the right. Increased fluorescent intensity (shown in red) for the breast cancer cases can be easily seen in several fractions.

Using these significant fractions, we constructed receiver operator characteristics (ROC) curves and area under the curve calculations for the top 5 statistically significant fractions. The ROC curves are based on the fluorescence values for each specific fraction from all patients with breast cancer and normal controls. After arranging the values from highest to lowest for a particular fraction, the intensity of each fluorescence value was plotted on a sensitivity vs. 1 – specificity graph as previously described (4). From this curve, the area

under the curve was calculated, which represents the predictive power of the autoantibody reaction for the fraction to distinguish between breast cancers from controls.

Figure 4 below illustrates our findings.

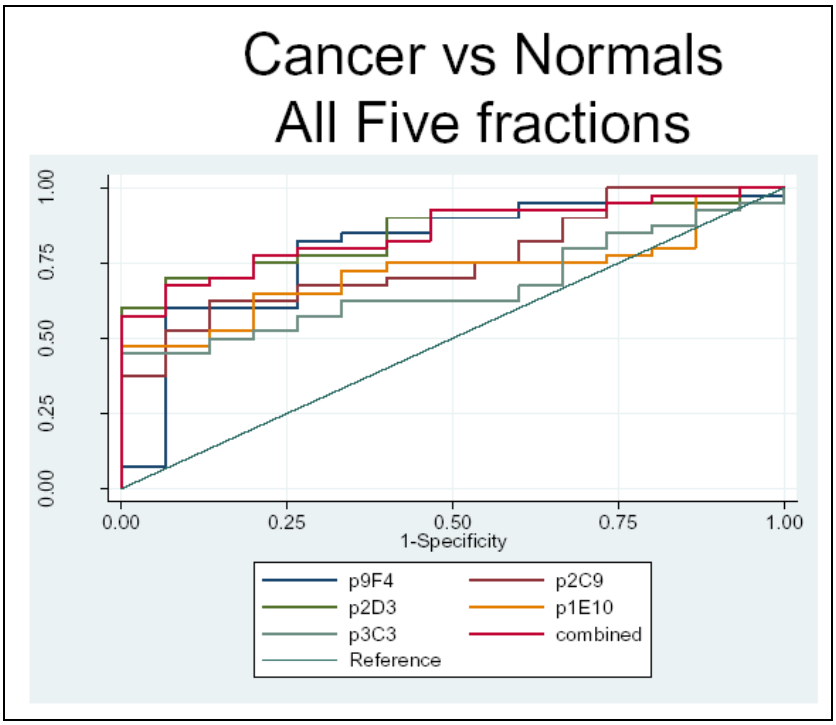

Figure 4: Figure 4 illustrates the ROC curves for the top 5 reactive fractions: p9F4, p2C9, p2D3, p1E10, and p3C3. A linear transformed model was used to obtain the ROC curve for the 5 fractions combined. The Y-axis is Sensitivity and the X-axis is 1 – Specificity. The area under the curve (AUC) for p9F4 is 0.79; p2C9 is 0.75; p2D3 is 0.85; p1E10 is 0.72; p3C3 is 0.67. The AUC for all 5 combined is 0.85. The reference line shows the expected plot at 50% sensitivity and 50% specificity.

We also separated our analysis with stage 1 breast cancers vs. normal controls and stage 2 breast cancers vs. normal controls. ROC curves and the area under the curve were performed. Figure 5 below illustrates our findings.

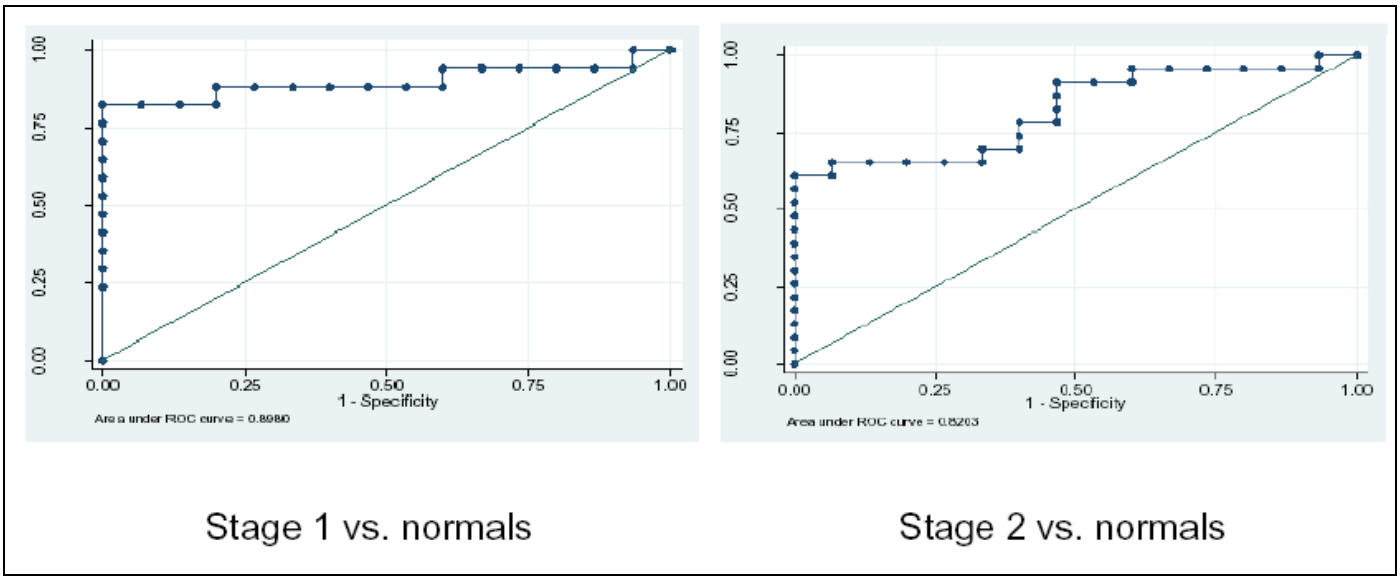

Figure 5: Figure 5 illustrates the ROC curves for the top 5 reactive fractions when we compared stage 1 breast cancers versus controls and stage 2 breast cancers versus controls. The top 5 reactive fractions (p9F4, p2D3, p3C3, p2C9, and p1E10), when combined, had an area under the curve of 0.898 for stage 1 breast cancer. The same fractions, when combined, had an area under the curve of 0.82 for stage 2 breast cancer. The reference line shows the expected plot at 50% sensitivity and 50% specificity.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a whole proteome native antigen fractionation microarray for breast cancer
- Identified significant antigen containing fractions
- Demonstrated that the top 5 antigen containing fractions have an area under the curve of 0.898 for stage 1 breast cancers versus normal controls and an area under the curve of 0.82 for stage 2 breast cancers versus normal controls
- Currently identifying the antigens in the fractions with mass spectrometry

REPORTABLE OUTCOMES:

Our reportable outcomes are the same as the key research accomplishments (above). In addition, we have an abstract that has been accepted for presentation for the 2010 ASCO-NCI-EORTC Annual Meeting on Molecular Markers in Cancer in Hollywood, FL (October 17-20, 2010). The abstract has not yet been published as the meeting has not yet taken place at the time of this report.

CONCLUSION:

Our work demonstrated that we have successfully developed a whole proteome native antigen fractionation microarray that can be used as a platform to profile autoantibody signatures for early stage breast cancer. In addition, we have identified antigen containing fractions that appears to have significant clinical utility for separating cancers from controls. Future work includes identifying the reactive antigens, validating our findings, preparing a manuscript for publication, and seeking additional funding.

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